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Calprotectin and Intestinal Fatty Acid Binding Protein (I-FABP) Level in Preterm Neonates with Necrotizing Enterocolitis

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KEYWORDS

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Abstract Necrotizing enterocolitis (NEC) is inflammatory state of intestinal tissue which mostly occurred in preterm neonates and associated with ischemia and inflammation. This study was aimed to investigate calprotectin level (inflammatory marker) and intestinal fatty acid binding protein (I-FABP) (ischemia marker) in preterm neonates with NEC. This research was designed as cross sectional which involve 32 preterm neonates divided into 2 groups as follows: NEC group (n=16 subjects) and control group (n=16 subjects). Diagnosis of NEC was established by clinical and radiological signs (abdominal distension and pneumatosis). Fecal calprotectin and urinary I-FABP were measured using ELISA method. Results showed that fecal calprotectin and urinary I-FABP was significantly higher in NEC group as compared to control group (*Mann-Whitney test*, $p<0.05$). Both calprotectin and I-FABP was positively correlated with NEC state (Spearman correlation test, $p=0.000$, $r=0.866$). Moreover, calprotectin and I-FABP level was positively correlated with grade (Bell's classification) and type (Gordon's classification) of NEC (Spearman correlation test, $p<0.05$). We concluded that calprotectin and I-FABP level was higher in NEC group. Moreover, I-FABP but not calprotectin, were correlated with grade and type of NEC.

Introduction

Necrotizing enterocolitis (NEC) is inflammatory state of intestinal tissue occurred mostly in preterm neonates which has various clinical symptoms characterized widely from mild mucosal damage to intestinal necrosis and perforation. Necrotizing enterocolitis is one of the major etiologies of morbidity and mortality in neonates especially in preterm and those with low birth weight (Neu, 2011).

The incidence of NEC is approximately 0.5%-3.5% of 1000 live birth and 13% in neonates low birth weight and 10% in term neonates, and mortality rate 20%-50% (Zvizdic *et al*., 2014). Incidence of NEC was negatively associated with gestational week and birth weight (Henry and Moss, 2009; Maheshwari *et al*., 2011).

Etiology of NEC is poorly understood. Some theories explain the pathophysiology of this gastrointestinal necrosis and perforation as the effect of impaired intestinal circulation, hypoxia and ischemia, inflammatory process, impact of type and volume of fluid intake, impact of normal flora and its colonization, intestinal maturity and immunity as well as genetic factor (Neu, 2011; Ahle *et al*., 2013).

Previous studies had been conducted to improve diagnostic procedure of NEC in early clinical stage in order to decrease its morbidity and mortality. The obstacles of this diagnostic procedure are difficulty to obtain blood sample, thereby various research conducted to establish non-invasive diagnostic procedure without blood sampling.

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Fecal calprotectin examination as inflammatory marker and urinary fatty acid binding protein (I-FABP) as intestinal ischemia marker in neonates with NEC are beneficial because of its non-invasive properties. Previous studies showed that fecal calprotectin and urinary I-FABP level was higher in NEC group compared with control and potential to be a biomarker for detecting early stage NEC in neonates. Thereby, this study was aimed to analyze the association of calprotectin and I-FABP in neonates with NEC. Furthermore, this study was designed to analyze the correlation of NEC grade and type with calprotectin and I-FABP level.

Materials and methods

Study Design

This study was designed as cross-sectional study to investigate the correlation of calprotectin and I-FABP level in NEC and control group. Furthermore, this study also investigated the correlation of calprotectin and I-FABP preterm neonates' level without NEC grade and type. This study involved 16 preterm neonates with NEC and 16 neonates as healthy control. Study was conducted at Neonatology Ward, Dr. Saiful Anwar General Hospital Malang and Biochemistry Laboratory, Faculty of Medicine, University of Brawijaya Malang. All procedures in this study have been approved by Ethical Committee of Research Dr. Saiful Anwar General Hospital Malang.

Research Subjects

Population target of this study was preterm neonates with low birth weight and NEC. This study included neonates hospitalized at Neonatology Ward, Dr. Saiful Anwar Hospital in October-December 2015. Sample used in this study were fecal obtained from preterm neonates with low birth weight and NEC. Subjects were included using consecutive sampling and meet the inclusion and exclusion criteria. Inclusion criteria for this study were described as follows: preterm neonates (<37 gestational weeks), low birth weight (<2500 grams), diagnosed as NEC and hospitalized at Dr. Saiful Anwar General Hospital Malang, and allowed by her/his family (informed consent). Exclusion criteria for this study were described as follows: preterm neonates with low birth weight which require surgical procedure and was not allowed by his/her family to join this study.

Measurement of Calprotectin Level

Fecal calprotectin level was measured by using ELISA methods (Human Calprotectin ELISA kit). Calprotectin level measurement could be performed after homogenization and extraction of sample using extraction buffer (0.1 M Tris, 0.15 M NaCl, 1.0 M urea, 10mM $CaCl_2$, 0.1 M monohydrate citric acid, 5 g/L BSA, 0.25 mM thimerosal (pH 8.0)). As many as 5 cc buffer was added into each 100 mg sample. The mixture was shaken for 20 minutes and centrifuged at 4°C for 20 minutes.

ELISA procedure for calprotectin level measurement was performed as instructed by manufacturer. Briefly, sample and standard were incubated in calprotectin-specific antibody. Antibody will bind to calprotectin, then conjugate with streptavidin-peroxidase. This conjugation will further be reacted with substrate tetramethylbenzidine. Enzymatic reaction could be stopped by adding oxalic acid. Absorbance of sample and standard were measured using spectrophotometry at wavelength 450 nm. Color intensity reflected the calprotectin level and by using standard graph, calprotectin level of each sample could be known.

Measurement of Intestinal Fatty Acid Binding Protein Level

Urinary I-FABP level was measured by ELISA methods as well (Human I-FABP ELISA kit). Urine samples were pretreated with centrifugation at 2000-3000 rpm for 20 minutes, then taking the supernatant for further analysis. ELISA procedure for I-FABP level measurement was performed as instructed by manufacturer. Briefly, sample and

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standard were incubated in I-FABP-specific antibody. Antibody will bind to I-FABP, then conjugate with streptavidin-peroxidase. This conjugation will further be reacted with substrate. Enzymatic reaction could be stopped by adding stop solution. Absorbance of sample and standard were measured using spectrophotometry at wavelength 450 nm. Color intensity reflected the calprotectin level and by using standard graph, calprotectin level of each sample could be known.

Statistical Analysis

Comparation study of fecal calprotectin and urinary I-FABP were performed using T-test or its alternative (Mann-Whitney). Correlation study was performed using Pearson correlation test or its alternative (Spearman). All statistic procedures were conducted at confidence interval 95% and considered as significant if p-value <0.05. All statistical procedures were performed using software SPSS for Windows version 24.0.

Results and discussions

Baseline Characteristics

This study was designed as cross sectional which involve 32 low birth weight neonates divided into 2 groups, NEC and control. Subject characteristics were demonstrated in Table 1.

Table 1. Subjects Characteristics

Subjects Characteristics	NEC Group (n=16)	Control Group (n=16)	Total	p-value
Sex				
Male	8/16	8/16	16	
Female	8/16	8/16	16	
Age				
Mean±SD (day)	6.75±0.7	2		0.003
Gestational Age				
Mean±SD (week)	34.25±3.17	32.87±3.34		0.001
Birth Weight				
Mean±SD (gram)	1796.6±398.2	1850±292.7		
Fetal Growth Status				
AGA (Appropiate for Gestational Age)	10/16	13/16	23	
SGA (Small for Gestational Age)	6/16	3/16	9	
Birth Place				
Dr. Saiful Anwar General Hospital Malang	4/16	9/16	13	
Other Hospital	12/16	7/16	19	
Apgar Score				
Minute 1 (mean±SD)	4.44±2.09	6.13±0.71		0.011
Minute 5 (mean±SD)	6.19±2.4	8.13±0.71		0.004
Mode of Delivery				
Vaginal delivery	7/16	10/16	17	
Abdominal delivery	9/16	6/16	15	
Maternal Risk Factor				
Hypertension	0	0	0	
Eclampsia	1/16	0	1	
Premature rupture of membrane	4/16	8/16	12	
Outcome				
Live	13/16	16/16	29	
Die	3/16	0	3	

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Difference of Calprotectin Level

The difference of calprotectin level between NEC groups and control group was analyzed using Mann-Whitney test. Figure 1 showed mean of calprotectin level in NEC and control group. Results showed that calprotectin levels were significantly higher in NEC group (521.2±106.29 ng/mL) as compared to control group (111.5±21.54 ng/mL) (p<0.001).

Figure 2 showed mean of calprotectin levels in each grade of NEC based on Bell's classification. Further analysis, showed in Figure 2, it suggested that there were no significant differences of calprotectin level between three grades of NEC based on Bell's classification (Kruskal Wallis, p=0.601).

Moreover, our finding in Figure 3 showed that calprotectin level in medical NEC (505.67±105.6 ng/mL) was not different as compared to surgical NEC (588.61±97.11 ng/mL) based on Gordon's classification of NEC (Mann Whitney, p=0.364). Figure 3 showed mean of calprotectin levels in surgical and medical NEC based on Gordon's classification.

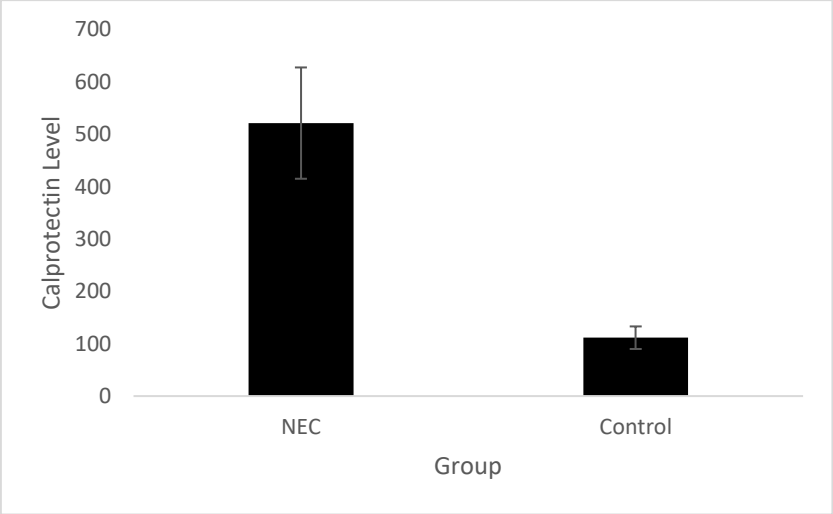

Figure 1. Fecal calprotectin level in NEC and control group.

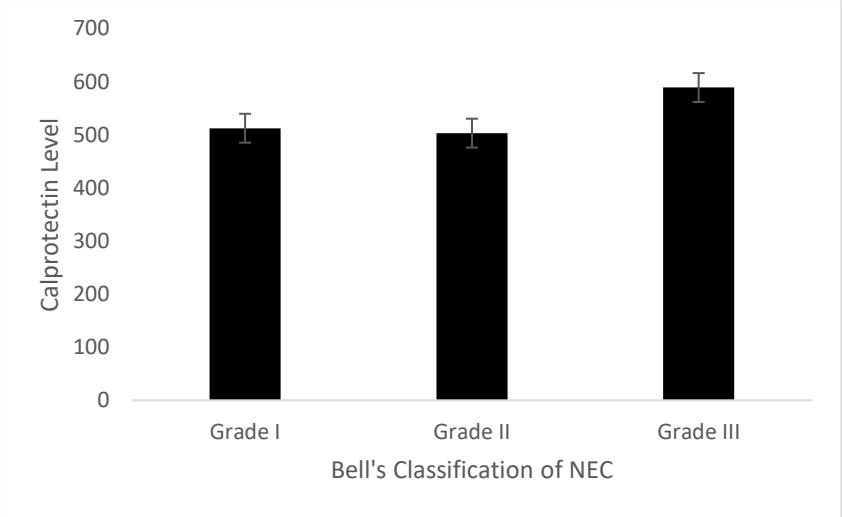

Figure 2. Fecal calprotectin level in each NEC grade based on Bell's classification.

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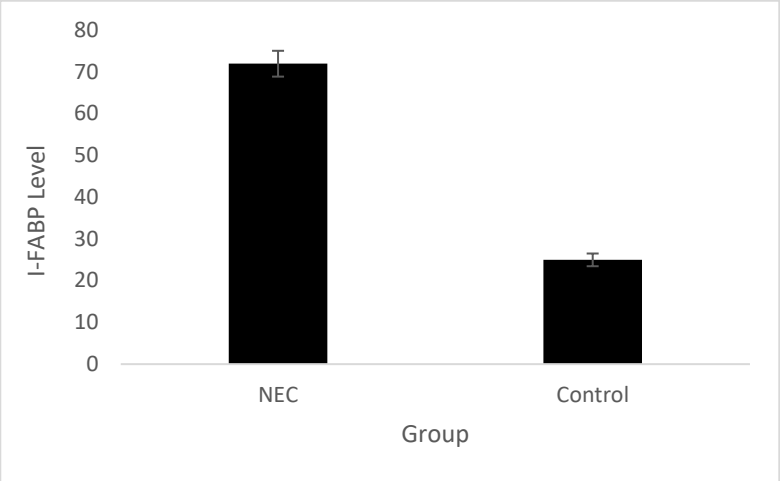

Figure 3. Fecal calprotectin level in medical and surgical NEC based on Gordon's classification.

Difference of I-FABP Level

The difference of I-FABP level between NEC groups and control group was analyzed using Mann-Whitney test. Figure 4 showed mean of I-FABP level in NEC and control group. Results showed that I-FABP levels was significantly higher in NEC group (71.9±3.09 ng/mL) as compared to control group (24.9±1.52 ng/mL) ($p<0.001$).

Data in Figure 5 showed mean of I-FABP levels in each grade of NEC based on Bell's classification. Further analysis, in Figure 5 showed that there were significant differences of I-FABP level between three grades of NEC based on Bell's classification (Kruskal Wallis, $p=0.028$). I-FABP level in grade III (75.26±3.26 ng/mL) was significantly higher compared with grade I (69.15±0.44 ng/mL) ($p=0.016$). I-FABP level in grade II (72.08±2.67 ng/mL) was not different as compared to grade I and grade III.

Moreover, our finding showed that I-FABP level in medical NEC (71.18±2.6 ng/mL) was not significantly different as compared to surgical NEC (75.26±3.26 ng/mL) based on Gordon's classification of NEC (Mann Whitney, $p=0.057$). Figure 6 showed mean of I-FABP levels in surgical and medical NEC based on Gordon's classification.

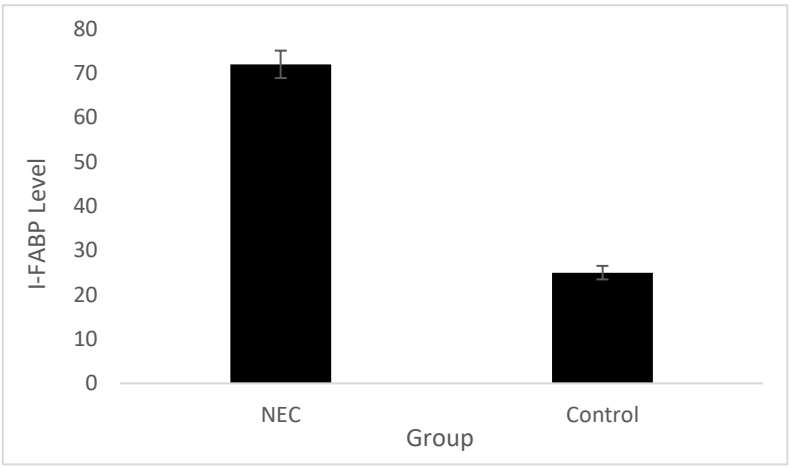

Figure 4. Urinary I-FABP level in NEC and control group.

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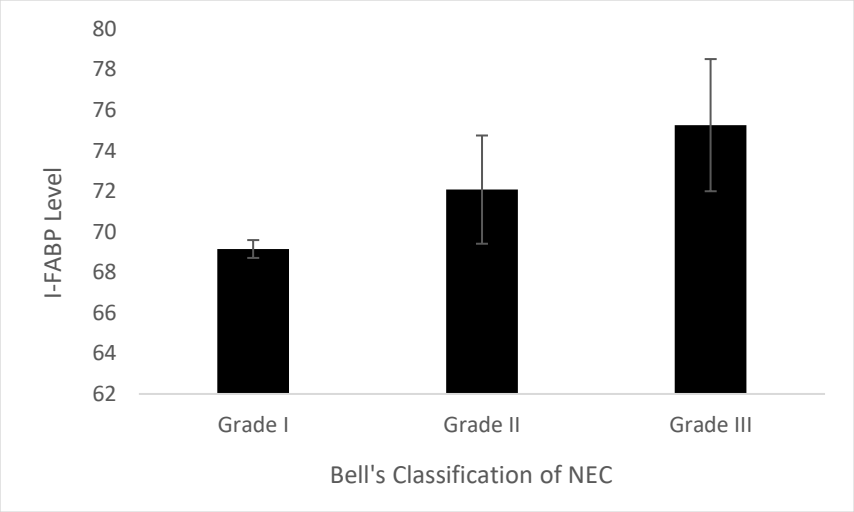

Figure 5. Urinary I-FABP level in each NEC grade based on Bell's classification.

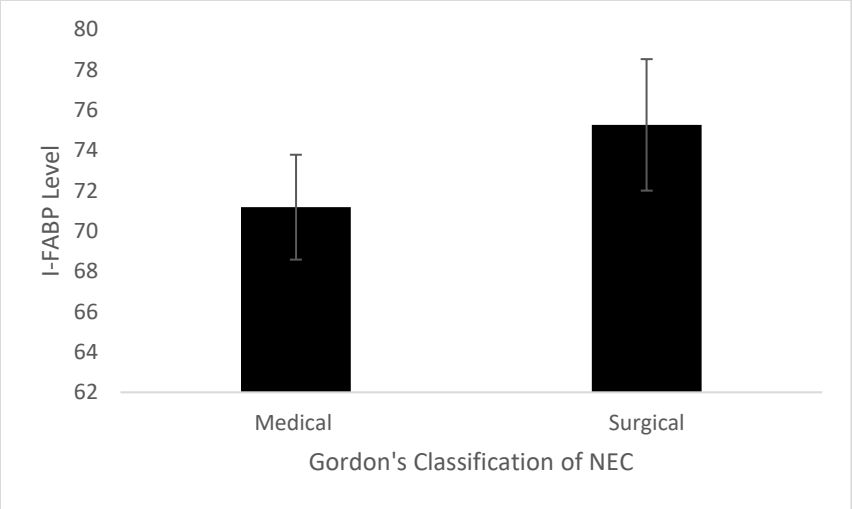

Figure 6. Urinary I-FABP level in medical and surgical NEC based on Gordon's classification.

Correlation of Calprotectin and I-FABP with NEC Status

Correlation study showed that fecal calprotectin level was positively correlated with NEC state (Spearman correlation test, p<0.001, r=0.866). However, in NEC group, fecal calprotectin level was not significantly correlated with NEC grade based on Bell's classification (Spearman correlation test, p=0.480, r=0.190) and NEC type based on Gordon's classification (Spearman correlation test, p=0.330, r=0.261).

Further analysis on I-FABP level showed that urinary I-FABP level was positively correlated with NEC state (Spearman correlation test, p<0.001, r=0.866). Interestingly, in NEC group, urinary I-FABP level was significantly correlated with NEC grade based on Bell's classification (Spearman correlation test, p=0.003, r=0.691) and NEC type based on Gordon's classification (Spearman correlation test, p=0.047, r=0.504).

Subject Characteristics

Our finding showed that in both NEC and control group, there were equal number of male and female neonates. This finding was similar with previous study which stated that NEC incidence was quite similar in male (55%) and

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female (45%) (Olariu *et al*., 2014). Diagnosis of NEC in our study was established in day 6^{th}-7^{th} after birth. This finding is also in accordance with Yee and colleagues which demonstrated that most NEC was diagnosed in average 6.7 days after birth. However, Clark and colleagues, using larger sample size, showed that median of age in which NEC established was at fifteenth days after birth in survivor group and eighteenth days in died group (Clark *et al.*, 2012).

Maternal factor also affects the NEC risk. In this study, mean of gestational age in NEC group was 34 weeks and control group were 32 weeks. Sidauruk and colleagues also found that NEC incidence was mostly from mother with gestational age 32 weeks (varied from 27-35 weeks) (Sidauruk *et al*., 2014). Multicenter study also showed that NEC incidence was higher in mother who deliver her baby at 34 weeks gestational age (Stout *et al*., 2008).

Mean of birth weight of neonates with NEC was 1796.56 grams and without NEC was 1850 grams. Sidauruk and colleagues demonstrated that NEC was occurred in 50% of 1000-1499 grams neonates and 30% in 1500-1999 grams neonates (Sidauruk *et al*., 2014). Incidence of NEC in Sweden was lower as compared to Sidauruk which showed about 183/10000 birth in group with birth weight 1000-1499 grams and only 22/10000 birth in group with birth weight 1500-2499 grams (Ahle *et al*., 2013). Further study showed that mean of birth weight of NEC group was 1078.19±338.72 grams (Olariu *et al*., 2014). This different finding might be caused by technological development of NICU in developed country.

Our findings also showed that 12 of 16 subjects with NEC were delivered in the other hospital/ health center. This phenomenon might be caused by several etiologies such as intrauterine infection, uncontrolled hypertension, untreated preeclampsia, and excessive enteral nutrition, as well as difficult labor. Decreased Apgar score in NEC group reflect hypoxia state in the early birth. Hypoxia particularly cause intestinal hypoxia which lead to NEC (Markel *et al*., 2014). Yee and colleague also showed that there was strong correlation between NEC and Apgar score (Yee *et al*., 2012). In this study, maternal risk factor was not associated with NEC incidence. This condition might be caused by limited subjects. Study about correlation of maternal risk factors such as hypertension, eclampsia, and premature rupture of membrane and NEC incidence had been studied (Samuels *et al*., 2017).

Fecal Calprotectin Level

Our study showed that calprotectin level was higher in NEC group compared with control group. Several studies also showed quite similar results (Albanna *et al.*, 2014, Aydemir, 2012). Yoon and colleagues showed that 16 neonates with very low birth weight hospitalized in NICU possess significantly higher calprotectin level in NEC group (Yoon *et al.*, 2014). Thuijls stated that calprotectin level could be used as biomarker for NEC with high sensitivity (86%) and specificity (93%) (Thuijls *et al.*, 2010). Other study also showed NEC neonates had higher calprotectin level (3000 µg/g) (Bin-Nun *et al*., 2015).

However, some studies also showed contradictory result. Selimoglu and colleagues showed that there was no difference between NEC group and control group. Several conditions such as intestinal damage and perforation could cause elevated calprotectin. Calprotectin also found higher in certain condition such as intestinal colic (Olafsdottir *et al.*, 2002) and milk allergy-related colitis (Burri and Beglinger, 2012).

Further analysis showed that there was no significant different calprotectin level based on grade (Bell's classification) and type (Gordon's classification) of NEC. Shenoy demonstrated that higher calprotectin level was correlated with clinical severity (Shenoy *et al*., 2014, Aydemir *et al*., 2012). However, Selimoglu showed that there were no differences between NEC grade I, II, and III (Selimoglu *et al*., 2012).

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Urinary I-FABP Level

Our findings showed that I-FABP in NEC group was higher in NEC group as compared to control group. Further study showed that grade III NEC has higher I-FABP level as compared to grade I NEC. However, I-FABP level in medical type NEC was insignificantly lower compared with surgical type NEC. This finding was in accordance with previous study which showed that I-FABP level was elevated for 7 days before onset of NEC (sensitivity 60%, specificity 78%) (Gregory *et al*., 2014). Reisinger and colleagues demonstrated that I-FABP was elevated in severe clinical condition of NEC and poor outcome (cut off 963 pg/ml could be used as outcome predictor, sensitivity 80% and specificity 94%) (Reisinger *et al*., 2014). Compared with sepsis condition, I-FABP was higher in NEC neonates (Coufal *et al*., 2016). Further analysis also showed that I-FABP was higher in grade III NEC as compared to grade II NEC, but could not differentiate medical and surgical type (Coufal *et al*., 2016). Urinary I-FABP level was significantly higher as compared to plasma level at 24 hours after diagnosis of NEC (Schurink *et al*., 2015). Other study showed that I-FABP was correlated with advanced stage of NEC, while L-FABP was correlated with suspected NEC (Evennett *et al*., 2010).

Conclusions

We concluded that calprotectin and I-FABP level was higher in NEC group. Moreover, I-FABP but not calprotectin, were correlated with grade and type of NEC.

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